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The essential and nonessential transcription units for viral protein synthesis and DNA replication of porcine circovirus type 2

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Abstract

During porcine circovirus (PCV) replication in PK15 cells, nine PCV type 2 (PCV2)-specific RNAs are synthesized. They include the capsid RNA (*CR*), five Rep-associated RNAs (*Rep, Rep′, Rep3a, Rep3b,* and *Rep3c*), and three NS-associated RNAs (*NS515, NS672,* and *NS0*). In this work, mutational analyses were conducted to investigate the involvement of each PCV2 transcription unit in viral protein synthesis and DNA replication. The results demonstrated that a stop codon introduced at the very 5′-end of *CR* did not affect Rep-associated antigens or viral DNA synthesis. Altering the consensus dinucleotides at the splice junctions of the minor RNAs (*Rep3a, Rep3b, Rep3c, NS515,* and *NS672*) or introducing a stop codon in the abundant *NS0* RNA also did not have any effect on viral protein synthesis or DNA replication. However, mutations that resulted in truncated Rep or Rep′ proteins caused greater than 99% reduction of viral protein synthesis and complete shut down of viral DNA replication. These results demonstrated that both *Rep* and *Rep′* are absolutely essential for PCV2 replication.

Keywords: Essential and nonessential genes of porcine circovirus type 2

Introduction

Porcine circovirus (PCV), psittacine beak-and-feather disease virus, goose circovirus, canary circovirus, and pigeon circovirus (Phenix et al., 2001; Ritchie et al., 1989; Todd et al., 1991, 2001; Woods et al., 1993) belong to the genus *Circovirus* of the *Circoviridae* family (McNulty et al., 2000; Pringle, 1999). The PCV virion is icosahedral, nonenveloped, and 17 nm in diameter (Tischer et al., 1982). Two genotypes of PCV have been identified. PCV type 1 (PCV1) was first detected as a contaminant of the porcine kidney PK15 cell line (CCL-33) distributed by the American Type Culture Collection (Tischer et al., 1974). PCV type 2 (PCV2) was initially identified in a Canadian swine herd in 1991 (Clarke, 1996; Harding, 1996). Serologic surveys indicated that both types of PCV are widespread in swine (Allan and Ellis, 2000; Segales and Domingo, 2002). Whereas PCV1 is nonpathogenic, PCV2 has been implicated as the etiological agent of a new disease, named postweaning multisystemic wasting syndrome (PMWS).

PCV has an ambisense, single-stranded, closed circular genome that encodes proteins by the encapsidated viral DNA, and by the complementary DNA of the replicative intermediate synthesized in the host. The genome sequences of a number of PCV1 and PCV2 isolates (Fenaux et al., 2000; Hamel et al., 1998; Meehan et al., 1997, 1998; Morosov et al., 1998; Niagro et al., 1998) have been determined. The overall DNA sequence homology within the PCV1 or PCV2 isolates is greater than 90%, while the homology between PCV1 and PCV2 isolates is 68–76%. Presence of a conserved stem-loop structure at the origin of DNA replication and similarities among the putative proteins essential for virus replication indicate that both PCVs are closely related to plant nanoviruses (Meehan et al., 1997; Niagro et al., 1998). Previous work demonstrated that 9 PCV2-specific RNAs (*CR, Rep, Rep′, Rep3a, Rep3b, Rep3c, NS515, NS672,* and *NS0*) and 12 PCV1-specific RNAs (*CR, Rep, Rep′, Rep3a, Rep3b, Rep3c-1, Rep3c-2,*

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Rep3c-3, *Rep3c-4*, *NS462*, *NS642*, and *NS0*) were detected during productive infection in PK15 cells (Cheung and Bolin, 2002; Cheung, 2003a,b). Members of the Rep-associated RNA cluster all share common 5′ and 3′ nucleotide (nt) sequences and they share common 3′ nt sequence with the NS-associated RNAs. *Rep*, capable of coding for the full-length Rep protein (Rep), appears to be the primary transcript that gives rise to the other Rep-associated RNAs by alternate splicing. The three NS-associated RNAs are probably transcribed from three different promoters present inside open reading frame 1 (ORF1), independent from the *Rep* promoter.

Based on nt sequence alignment analysis of the PCV1 and PCV2 RNAs, *CR*, *Rep*, *Rep′*, *Rep3a*, *Rep3b*, and *NS0* are equivalent entities for the two viruses. However, quantitative and qualitative differences were observed among *Rep3c* and the NS-associated RNAs (Cheung, 2003b). Whether these genetic differences contribute to PCV2 pathogenicity remains to be elucidated. In this work, mutagenesis studies were conducted with the PCV2 genome to determine the essential and nonessential transcription units involved in viral protein synthesis and DNA replication.

Results

Experimental design

The PCV2 genome was cloned into the *Bam*H1 site of Bluescript plasmid (Strategene, San Diego, CA) after modifying nt 1015–1020 (taatgg to ggatcc) (GenBank Accession No. AY094619) into a *Bam*H1 site to generate plasmid Y1. Y1, designated the "parent," was used to construct all other PCV2 mutant plasmids. Specific mutation was introduced into each transcription unit using the QuickChange Site-Directed Mutagenesis Kit (Strategene) with predetermined oligonucleotide primers. The following two types of mutation were engineered (Fig. 1a): (1) Donor splice junction mutation: the consensus dinucleotide (i.e., GT) at the inner boundary of a donor spliced site (GT. . .AG) was altered. Presumably, the targeted spliced RNA would not be generated. (2) In-frame termination mutation: a stop codon was engineered into the coding sequence of a specific ORF without causing any amino acid (aa) change in other overlapping ORFs. Presumably, a truncated protein specific to the affected ORF would result from each mutation. Since transfection with Y1 plasmid containing the Bluescript vector (Strategene) consistently gave very few PCV-antigen producing cells (data not shown), the experiments were carried out with plasmid DNAs that were digested with *Bam*H1 and religated to circularize the viral genome prior to transfection into PK15 cells. At 48 h posttransfection, the cultures were assayed for viral protein synthesis by immunochemical staining with a hyperimmune swine antiserum raised against PCV2 (B6) or a rabbit antiserum raised against the capsid protein of PCV2 (R1) (Cheung and Bolin, 2002; Nawagitgul et al., 2000), and for newly synthesized viral DNA by PCR before and after *Dpn*1 digestion (see Materials and methods). There were five *Dpn*1-sensitive sites between nt 1447 and 865 in the input PCV2 DNAs propagated in *Escherichia coli* (Fig. 1a).

Donor splice junction mutation for Rep3a, Rep3b, Rep′, NS515, and NS672

For *Rep3* mutant plasmid (S98), the dinucleotide at splice junction nt 97 was changed from GT to AT (nt 98) (Fig. 1a). This change was expected to prevent the generation of all *Rep3* RNA species (i.e., *Rep3a*, *Rep3b*, and *Rep3c*) but not cause any aa change in Rep or Rep′. For the *Rep′* mutant plasmid (S418), two point mutations were introduced around splice junction nt 416. At nt 416, T was changed to A and it was a silent mutation for Rep. At nt 418, the consensus dinucleotide GT was changed to GC. It was not possible to introduce a silent mutation in the consensus dinucleotide of this splice junction and this mutation changed a Rep aa residue from valine to alanine. For the *NS515* and *NS672* mutant plasmids (S517 and S674, respectively), the dinucleotide at splice junctions nt 515 and 672 were changed from GT to GC (nt 517) and from GT to GA (nt 674), respectively. Although both *NS515* and *NS672* do not appear to code for any functional proteins (Cheung, 2003a), these mutations eliminated any function they may have by preventing their formation. These two changes were not in the coding region of *Rep′* and they did not introduce any aa residue change in Rep.

To ensure that the splice RNAs were not synthesized by the mutant plasmids, presence of *Rep′*, *Rep3a*, *Rep3b*, *NS515*, and *NS672* in the transfected cultures was assayed by RT-PCR with predetermined oligonucleotide primers. These primers were located on either side of the splice junction. In addition, prior to RT-PCR amplification, the RNA samples were digested with DNase 1 (e.g., D1) to remove any contaminating viral DNA (compare lanes D1 and Y1 of Fig. 2a). Amplification with primers 47F and 890R (Fig. 2a) showed that Y1, S517, and S674 synthesized *Rep′* (○) and *Rep3a* and *Rep3b* (△); S98 synthesized *Rep′* but not *Rep3a* or *Rep3b*; and S418 synthesized *Rep3a* and *Rep3b* but not *Rep′*. *Rep3c* is a rare RNA (Cheung, 2003a) and it was not apparent in the current transfection experiment. From the fact that S98 did not synthesize *Rep3a* or *Rep3b*, it is logical to conclude that *Rep3c* was not synthesized by S98 either. Amplification with primers 466F and 995R (Fig. 2b) showed that Y1 and S674 synthesized both *Rep* (×) and *NS515* (◇), and S517 synthesized *Rep* but not *NS515*. Amplification with primers 610F and 995R (Fig. 2c) showed that Y1 and S517 synthesized both *Rep* or *NS0* (×) and *NS672* (▽), and S674 synthesized *Rep* or *NS0* but not *NS672*. These results demonstrated that the mutation introduced at the splice junction abrogated the synthesis of each specific RNA without affecting others.

Viral protein synthesis in the transfected cultures was assayed by immunochemical staining with B6 antiserum.

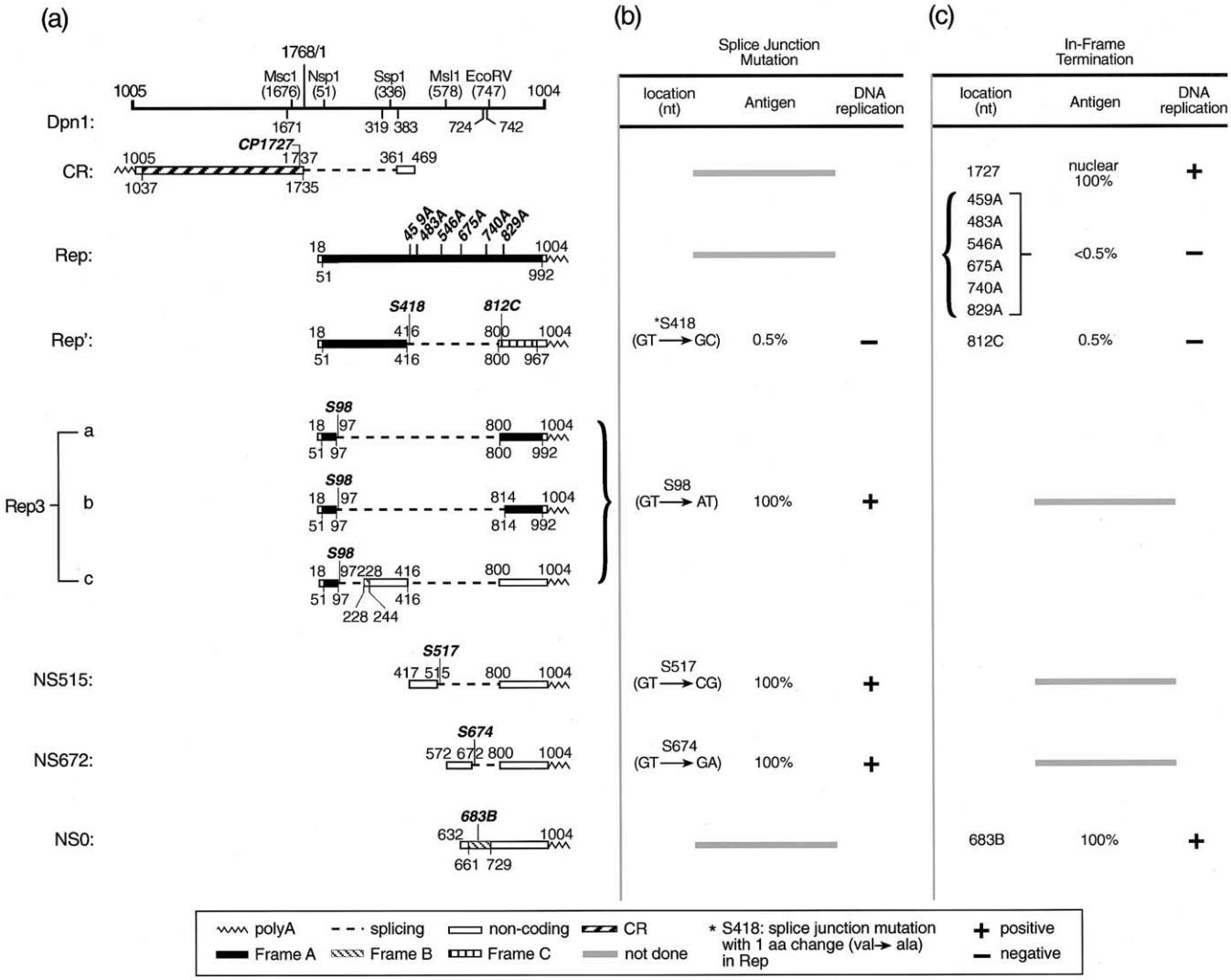

Fig. 1. (a) Schematic representation of the PCV2/688 genome and the specific mutations introduced. The nucleotide sequence was based on GenBank Accession No. AY094619. *CR* is transcribed leftward and the rest are transcribed rightward. The RNAs are annotated with nt coordinates that indicate the last nucleotide of each respective exon. The coding sequence of each transcript was shaded and their nt coordinates are indicated below each RNA. The *Dpn*1 restriction enzyme sites are also indicated. (b) Summary of splice junction mutations. (c) Summary of in-frame mutations.

An abundant number of cells exhibiting cytoplasmic and nuclear viral proteins were observed with Y1, S98, S517, and S674, while few viral antigen-positive cells (0.1% of Y1) were detected with S418 (Fig. 3).

Newly synthesized viral DNA was assayed by PCR with primers 1447F and 865R before and after the samples were digested with *Dpn*1 restriction enzyme. *Dpn*1-resistant viral DNAs were detected with cells transfected with Y1, S98, S517, and S674, but not with the mock- or S418-transfected cells (Fig. 4).

In-frame termination mutation

A stop codon was introduced into the coding sequence of *CR*, *Rep*, *Rep′*, or *NS0* (Fig. 1a). Each mutation was specific to an ORF without affecting others and caused early termination of the polypeptide coded by that specific RNA. The mutation was designated by the RNA, the nt position, and the reading frame (if necessary) it affected. In a previous article (Cheung, 2003a), the entire *Rep* was assigned Frame A; the 3′ portion of *Rep′* was assigned Frame C, and the coding sequence of *NS0* was assigned Frame B (Fig. 1a). For *CR* mutant (CP1727), nt 1727 was changed from TAT to TAA; for *Rep* mutant (Rep459A), nt 459 was changed from GAG to TAG; for *Rep′* mutant (Rep′812C), nt 812 was changed from CAG to TAG; and for *NS0* mutant (NS0683B), nt 683 was changed from TGG to TGA.

An abundant number of viral antigen-positive cells (stained with B6 antiserum) were detected with cultures transfected with Y1, CP1727, and NS0683B (Fig. 5) and the number of antigen-positive cells in these cultures was essentially the same. However, the antigens in CP1727-transfected cells were predominantly nuclear, as opposed to

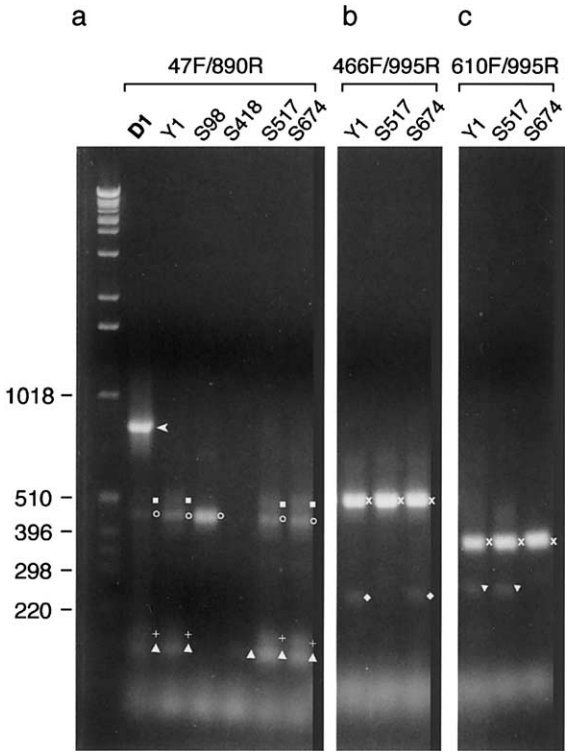

Fig. 2. Detection of splice RNAs in transfected cultures by RT-PCR. (a) Primers 47F and 890R were used. (b) Primers 466F and 995R were used. (c) Primers 610F and 995R were used. The plasmid used for each transfection is indicated on top of each lane. The PCR product derived from contaminating viral DNA is indicated by (◀). PCR products derived from *Rep* or *NS0* are indicated by (×), products derived from *Rep′* are indicated by (○); products derived from *Rep3a* and *Rep3b* are indicated by (△); products derived from *NS515* are indicated by (◇); products derived from *NS672* are indicated by (▽). Hybrids of *Rep3a*/*Rep3b* PCR products are indicated by (+), and hybrids of *Rep′*/*Rep3a* and *Rep′*/*Rep3b* PCR products are indicated by (□) (Cheung, 2003a).

being both cytoplasmic and nuclear with Y1 and NS0683B. Since the CP1727 nuclear antigens were not recognized by the CP-specific R1 antiserum (data not shown), these antigens were probably derived from the Rep region. In contrast, cultures transfected with Rep459A and Rep′812C, which also stained with B6 serum, showed less than 1% viral antigen-positive cells as compared to Y1. When newly synthesized viral DNA was assayed by PCR with primers 1236F and 427R before and after the samples were digested with *Dpn*1, *Dpn*1-resistant viral DNA was detected with Y1, CP1272, and NS0683B but not with Rep459A or Rep′812C (Fig. 6).

Full-length Rep is essential for viral protein synthesis and DNA replication

Rep of PCV2 contains the P-loop NTP-binding domain and three conserved motifs that have been identified in PCV1 (Mankertz et al., 1998a) and in other rolling circle

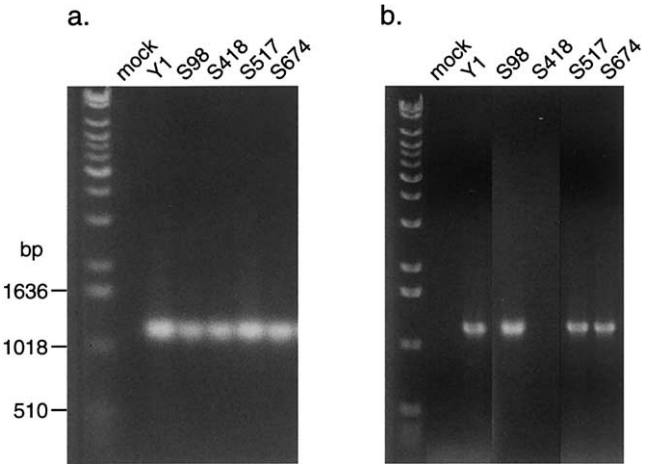

Fig. 4. *Dpn*1 assay for spliced junction mutant plasmid experiment. (a) PCR assay prior to *Dpn*1 digestion. (b) PCR assay after *Dpn*1 digestion. The plasmid used for each transfection is indicated on top of each lane.

replication (RCR) initiator proteins (Ilyina and Koonin, 1992) designated rcr-Rep in this article. Experiments similar to those conducted with PCV1 (Mankertz and Hillenbrand, 2001) were set up to investigate whether a truncated PCV2 Rep that contains these RCR motifs was sufficient for PCV2 replication and to examine the importance of the P-loop domain.

3′ truncation

Termination codons were introduced at nt 106–107A (TTC to TAA), 459A (GAG to TAG), 483A (AGA to TGA), 546A (AAG to TAG), 675A (GAA to TAA), 740A (TGT to TGA), and 829A (TTG to TAG). The mutation at nt 106–107A, located before the three RCR motifs, affected both Rep and Rep′, but not Rep3a or Rep3b. The 459A, 483A, and 546A mutations were located after the three replication motifs but before the P-loop domain. The 675A

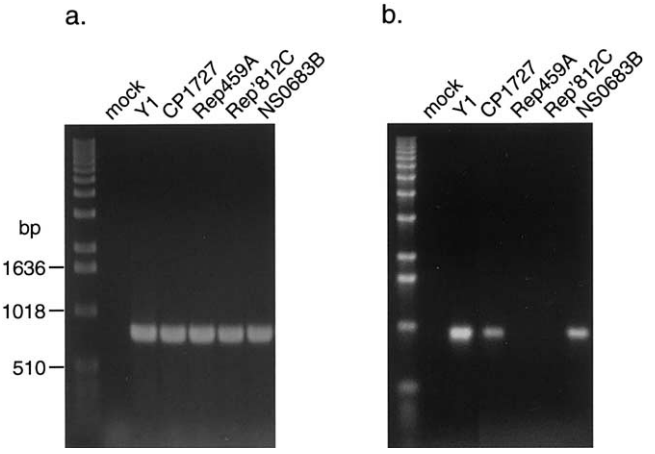

Fig. 6. *Dpn*1 assay for in-frame mutant plasmid experiment. (a) PCR assay prior to *Dpn*1 digestion. (b) PCR assay after *Dpn*1 digestion. The plasmid used for each transfection is indicated on top of each lane.

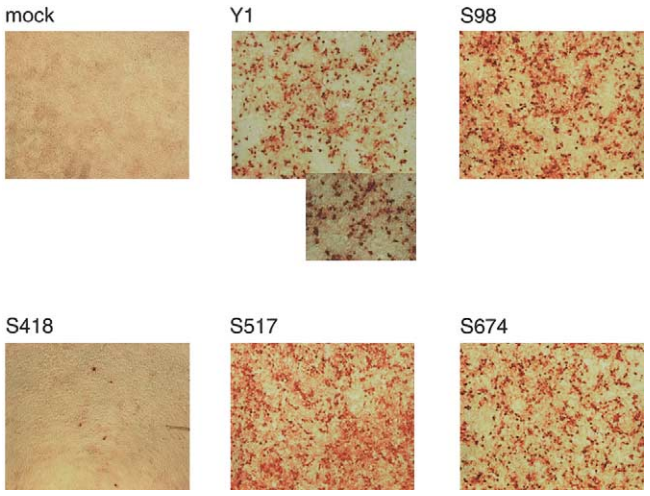

Fig. 3. Immunochemical staining of PK15 cells transfected with PCV2 splice junction mutant plasmids. The plasmid used for each transfection is indicated on top of each panel.

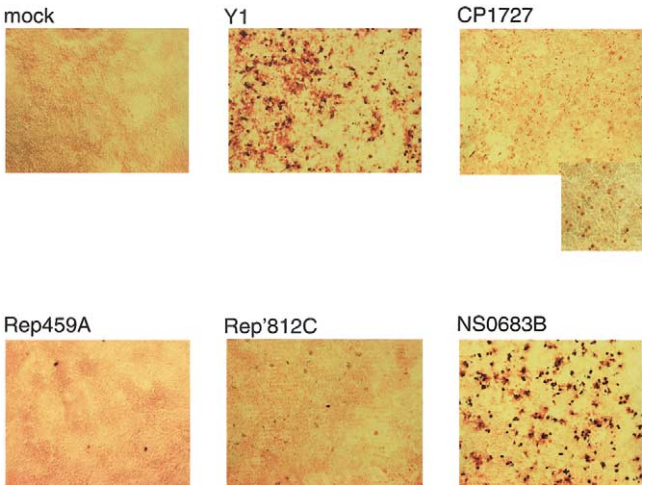

Fig. 5. Immunochemical staining of PK15 cells transfected with PCV2 in-frame mutant plasmids. The plasmid used for each transfection is indicated on top of each panel.

and 740A mutations were located after the P-loop motif and they did not affect NS0. The 829A mutation affected Rep, Rep3a, and Rep3b but not Rep′. Whereas an abundant number of viral antigen-positive cells and *Dpn*1-resistant viral DNA were detected with Y1, the number of viral antigen-positive cells from all the mutant plasmids in this experiment was reduced to less than 0.5% of Y1 and viral DNA replication was not detected (data not shown).

P-loop modification

The PCV2 Rep protein contains a short stretch of glycine-rich sequence ending with glycine-lysine-serine (GKS) to form a P-loop NTP-binding domain. In this experiment, the PCV2 P-loop core motif (GKS) located at aa 179–181 of the deduced PCV2 Rep was mutated to DIS. After transfection into PK15 cells, only a small number of PCV2-antigen expressing cells (approximately 0.3% of Y1) were observed and viral DNA synthesis was not detected with this DIS plasmid (data not shown).

Reduction of viral capsid protein synthesis in the absence of functional Rep and Rep′

An experiment was set up to investigate whether PCV2 capsid protein is expressed in the presence of defective Rep or Rep′. Equivalent amounts of recircularized Y1, Rep459A, and Rep′812C were transfected into parallel cultures. At 48 h posttransfection, the cultures were immunochemically stained with B6 or the CP-specific R1 antiserum. Whereas an abundant number of cells transfected with Y1 were stained with both sera, few cells were stained with either B6 or R1 serum (less than 0.5% of Y1) in the Rep459A and Rep′812C transfected cultures (Fig. 7).

Discussion

Previous work showed that nine RNAs, *CR*, *Rep*, *Rep′*, *Rep3a*, *Rep3b*, *Rep3c*, *NS515*, *NS672*, and *NS0* were synthesized during productive infection of PCV2 in PK15 cells (Cheung, 2003a). In this work, mutagenesis experiments were conducted to evaluate the involvement of each RNA in viral protein synthesis and DNA replication. The results are summarized in Fig. 1. Plasmids with early termination or splice junction mutations in *CR*, *Rep3a*, *Rep3b*, *Rep3c*, *NS515*, *NS672*, and *NS0* that rendered the encoded protein nonfunctional exhibited similar numbers of PCV-antigen expressing cells as the parent Y1 plasmid and they were capable of synthesizing *Dpn*1-resistant PCV2 DNA. Thus, these transcription units are nonessential for PCV2 replication. In contrast, only a small percentage (less than 1% of Y1) of the cells transfected with *Rep* or *Rep′* mutant plasmids were positive for viral antigens and viral DNA repli-

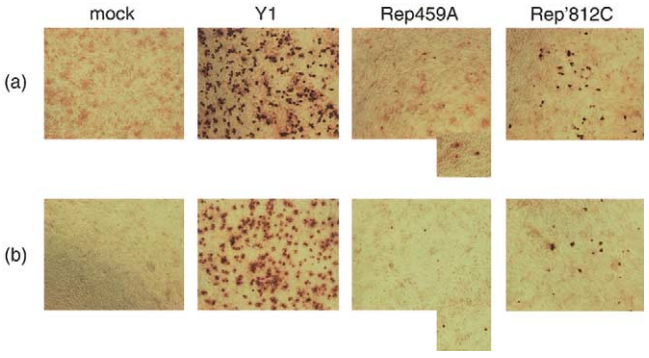

Fig. 7. Capsid protein expression in the presence of defective Rep or Rep′. (a) Immunochemical staining of transfected cultures with B6 serum. (b) Immunochemical staining of transfected cultures with CP-specific R1 serum. The plasmid used for each transfection is indicated on top of the panel.

cation was not detected. Therefore, both *Rep* and *Rep′* are essential for efficient viral protein and DNA synthesis. These observations are in agreement with results obtained from a transient expression system that indicated PCV1 Rep and Rep′ are required for PCV1 DNA replication (Mankertz and Hillenbrand, 2001) and support the binding assay data that demonstrated physical interactions between Rep and Rep′ with the viral genome at the origin of viral DNA replication (Steinfeldt et al., 2001).

Except for PCV1 and PCV2, all other known examples of DNA synthesis via the RCR mechanism utilize only one multifunctional rcr-Rep (Hanley-Bowdoin et al., 2000). Similar to rcr-Rep, the Rep proteins of PCV1 and PCV2 also contain the P-loop NTP-binding domain and the three conserved RCR motifs (Gutierrez, 1999; Ilyina and Koonin, 1992; Mankertz et al., 1998a). It is interesting to note that the Mastreviruses of the *Geminiviridae* family also synthesize two RNAs, *rcr-Rep* and *RepA*, by alternate splicing; as such, they share common 5′ and 3′ nt sequences. The *rcr-Rep* is a spliced RNA that codes for rcr-Rep, a protein similar to PCV Rep that includes the three conserved RCR motifs and the P-loop domain. *RepA* is an unspliced RNA that codes for a protein (similar to PCV Rep′) that includes the three conserved RCR motifs and a 3′ aa sequence different from the 3′ aa sequence of Rep. For Mastreviruses, only rcr-Rep is essential for viral DNA replication. Therefore, it is unexpected to find that PCV1 and PCV2 require two proteins, both Rep and Rep′, for viral DNA replication (Mankertz and Hillenbrand, 2001; this study).

Previous work showed that the P-loop and RCR motifs were essential functional domains of PCV1 Rep (Mankertz and Hillenbrand, 2001). In this work, plasmids Rep675A and Rep740A, which contain the P-loop domain as well as the three RCR motifs, were not capable of self-DNA replication. Also, these mutant plasmids were not complemented by Rep3a or Rep3b, which contain 3′ aa sequence identical to Rep. Therefore, not only do these results emphasize the importance of the 3′ portion of Rep, they also indicate that a full-length and intact Rep is necessary for PCV protein synthesis and DNA replication. In fact, several conserved motifs downstream of the P-loop domain have been noted among the Rep proteins of PCV1, PCV2, psittacine beak-and-feather disease virus, goose circovirus, canary circovirus, and pigeon circovirus (Phenix et al., 2001).

It has been demonstrated that the PCV1 *CR* promoter is transcribed by cellular enzymes when cloned into a reporter plasmid (Mankertz et al., 1998b). This observation is supported by the PCV2 mutant plasmids Rep459A and Rep′812C experiment, which showed that CP was detected in cells of a specific physiologic state in the absence of functional Rep and Rep′ proteins (Fig. 7). As demonstrated, when PK15 cells were transfected with equivalent amounts of Y1, Rep459A, and Rep′812C, only a small number of CP-positive cells (less than 0.5% of Y1) were observed with mutant plasmids Rep459A or Rep′812C, and that number was greatly enhanced in the presence of functional Rep and Rep′ (Y1) (Fig. 7). Although it has been reported that the PCV1 *CR* promoter is not influence by any viral encoded proteins (Mankertz and Hillenbrand, 2002), whether the CP expression enhancement observed with PCV2 in this study is the sole result of genome amplification following DNA replication or that PCV2 Rep and Rep′ have some influence on the *CR* promoter remains to be elucidated.

Materials and methods

Virus, cell, and serum

A PCV2 isolate (PCV/688) that had been shown to induce PMWS in pigs (Bolin et al., 2001), a hyperimmune swine serum against PCV2 (B6), and a rabbit antiserum raised against the capsid protein of PCV2 (R1) were used (Cheung and Bolin, 2002; Nawagitgul et al., 2000). PCV1-free PK15 cell lines were maintained in MEM–Hank's balanced salt solution (MEM-H) (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum.

Oligonucleotide primers

The primers for genome amplification were identified by the first nt coordinate of each oligomer with respect to the genomic sequence and they were numbered according to the PCV2 coordinates (GenBank Accession No. AY094619). The suffix (F or R) of the oligonucleotide indicates the orientation of the primer. F indicates forward direction from nt 1 to 1768, while R indicates reverse direction from nt 1768 to 1. The primers are as follows: 47F: CAACATGCCCAGCAGGAAGAATGGAAG; 466F: ACCCTGTAACGTTTGTCAGAA; 610F: TTGCAGACCCGGAAACCAC; 1236F: AGTACAGGTTTGGGTGTG; 1447F: TGTAGTATTCAAAGGGTATAGAGA; 427R: AAGGTACTCACAGCAGTAGACAGGTC; and 865R: TCTACAGCTGGGACAGCAGTTG; 890R: GGAAGTAATCCTCCGATAGAGAGC; 995R: CTCAGTAATTTATTTCATATGG.

The primer sets for mutagenesis are identified by the nature and location of the mutation with respect to the genomic sequence. Oligonucleotides with prefix S are splice junction mutation primers and the in-frame mutation primes are indicated with respect to the RNA. Only one strand of the mutagenic primer set is listed and the mutation nt is indicated in uppercase letters, as follows: S97: ccacataaaagAtgggtgttcac; S418: gacctgtctactgcAgCgagtaccttgttg; S517: gaacttttgaaagCgagcgggaaaaatg; S674: ggttaccatggAgaagaagtggttgtt; CP1727: gcctccttggTtacgtcatag; Rep106–107: aaaggtgggtgtAAacgctgaataat; Rep459A: gaccgttgcaTagcagcaccc; Rep504A: ccgcgggctggctTaacttttgaaagt; Rep546A: gaagcgtgattggTagaccaatgtaca; Rep675A: ggttaccatggaTaagaagtggttgtt; Rep740A: ctactgagactgtgAgatcgatatccattg; P-loop: gccacctgggtgtgAtaTaagcaaatgggctg; NS0683B: tgaa-

gaagtgAttgttattga; Rep′812C: ttctgattacTagcaatcaga; and Rep829A: cagaccccgtAggaatggtac.

DNA mutagenesis and transfection

Specific mutation was introduced into the cloned PCV2 genome using the QuickChange Site-Directed Mutagenesis Kit (Strategene). Mutant plasmids were generated with each mutagenesis primer set according to the manufacturer's instructions. After the PCV2 genome was excised from the Bluescript plasmid and circularized by ligation, the ligated DNA mixture was transfected into PK15 cells which had been seeded into 48-well tissue culture plates such that approximately 60–80% confluency was reached 24 h later. Transfection of the ligated DNA mixture (0.5 μg) was carried out with a commercially available lipofectamine reagent (30 μg/ml) in MEM-H. The DNA/lipofectamine mixture (0.3 ml) was dispensed into each culture which had been freshly rinsed with MEM-H. After incubation for 5 h at 37 C, the DNA/lipofectamine mixture was replaced with MEM-H with 10% fetal bovine serum.

Immunochemical staining

PK15 monolayer cells seeded in 48-well culture plates were infected with virus or transfected with DNA. At 48 p.i., the cells were rinsed with water, fixed in a PBS solution containing 40% acetone and 0.2% BSA (−20°C) for 10 min, and dried for 30 min at 37°C. The cells were then incubated with anti-PCV serum diluted in binding buffer (0.01% Tween 20 and 0.5 M NaCl in PBS) for 1 h at room temperature, washed twice with PBS containing 0.05% Tween 20 (PBSW), incubated with Protein G conjugated with horseradish peroxidase (1:1000) (Zymed Labs, Inc., San Francisco, CA) for 30 min, and rinsed with PBSW twice. Color development was carried out with 3-amino-9-ethylcarbazole (AEC) and hydrogen peroxide in 0.05 M sodium acetate buffer (pH 5). Viral antigens were stained reddish-brown in this assay.

RNA and DNA

Total cell RNAs or DNAs were isolated using the STAT-60 RNA or STAT-60 DNA extraction kit purchased from TEL-TEXT "B", Inc. (Friendswood, TX). The cells were lysed and extracted with chloroform according to the manufacturer's instructions. The RNA samples were incubated with DNase 1 for 60 min at 37°C to remove any contaminating viral DNA and the DNA samples were incubated with *Dpn*1 at 37°C overnight to digest the input plasmid- derived viral DNA prior to amplification with predetermined oligonucleotide primers. The PCR product was electrophoresed in 1.2% agarose gel and the DNA patterns were photographed after staining with 0.05 mg/ml ethidium bromide.

PCR

RT-PCR was carried out with 1 μg of RNA in the presence of 10 mM Tris–HCl (pH 8.3), 0.2 mM each of dNTP, 100 pM each of the upstream and downstream primer, 2.5 U of Taq polymerase, 50 U of MMTV reverse transcriptase, and 20 U of RNAsin in 50 μl. The reaction mixture was first incubated at 50°C for 30 min and then amplified for 45 cycles at 94°C (10 s), 55°C (30 s), and 70°C (30 s). DNA-PCR was carried out under identical conditions with omission of the reverse transcriptase, RNAsin, and 30 min 50°C incubation.

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